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Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

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PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts:  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

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**Blatt 2 der Bescheinigung**  
**Sheet 2 of the certificate**  
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The application was transferred from the original applicant  
GIST-BROCADES B.V. to the above-mentioned applicant on 14.03.96.  
The original title of the invention reads as follows:  
"Novel cellulase and its applications".

It is further known that detergent compositions comprising cellulases are effective in removing dirt (cleaning). The efficiency of cellulolytic enzymes, cellulases, in terms of cleaning textile has been recognized for some time; GB-A-2075028, GB-A-2095275 and GB-A-2094826 (Kao Corporation) disclose detergent compositions with cellulase for improved cleaning performance.

It is also known in the art that cellulases can act as a colour clarifying agent in laundry detergents. After repeated washing of soiled fabrics, cotton containing fabrics appear to be greyish, most probably due to disrupted fibres caused by mechanical action and causes the greyish appearance of coloured cotton containing fabrics. The fibres are torn up resulting in disordered fibres which are broken. The use of cellulases as colour clarification agents for coloured fabrics has been described in EP-A-0220016 (Novo-Nordisk).

The main disadvantage of the cellulases known in the art showing colour clarification is that these enzymes aggressively degrade the cellulose containing fabrics which results in undesirable loss of tensile strength of the fabrics.

On the other hand cellulases known to the art showing good cleaning properties show hardly any colour clarification effects.

From the above it will become clear that it is still desirable to provide for improved cellulases in detergent applications.

### Summary of the invention

The present invention relates to a novel (single) cellulase which cellulase exhibits the following properties:

(a) show a delta REM of at least 4 units in the Anti Redeposition test, and

(b) show a depilling result which depilling result is at least comparable to that of the cellulase obtainable from Bacillus sp. CBS 670.93 in the Depilling test.

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GIST-BROCADES B.V.

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NOVEL CELLULASE AND ITS APPLICATIONS

Technical field

The present invention relates to a novel cellulase. The invention further relates to detergent additives comprising the novel cellulase and to detergent compositions containing the novel cellulase. The invention also relates to the use of the novel cellulase in the treatment of cotton containing fabrics.

Background of the invention

Cellulases, also called cellulolytic enzymes, are enzymes which are capable of the hydrolysis of the  $\beta$ -D-glucosidic linkages in celluloses. Cellulolytic enzymes have been divided traditionally into three classes: endoglucanases, exoglucanases or cellobiohydrolases and  $\beta$ -glucosidases (Knowles, J. et al. (1987), TIBTECH 5, 255-261). Cellulolytic enzymes can be produced by a large number of bacteria, yeasts and fungi. Microorganisms that produce cellulases are for example described in GB 2094826 (Kao Corporation).

Several applications have been developed for use of cellulolytic enzymes:

- degrading (wood)cellulose pulp into sugars for (bio)ethanol production;
- several textile treatments like 'stone washing' and 'biopolishing';
- application in detergent compositions.

The use of cellulases in detergent compositions started with cellulases capable of reducing the harshness (softening) of cotton containing fabrics (GB 1358599 (Unilever)).

Surprisingly it has been found that there are (single) cellulases which are capable of both cleaning, antiredeposition, colour clarification (by depilling action of the cellulase) and pilling prevention (antipilling) performance in laundry washing, obtainable from microorganisms.

Mixtures of cellulases as suggested in WO 95/02675 (Novo and Procter & Gamble) and known mixtures of cellulases like Celluzyme® (Novo) were known to provide the above mentioned performances in laundry washing, but single enzymes providing all these characteristics when applied in laundry washing are novel.

It is further found that the (single) cellulase of the invention, unlike previously known mixtures of cellulases which provide colour clarification, do not degrade cotton to an undesirable level causing tensile strength loss.

It is further found that this cellulase unlike previously known cellulases which provide colour clarification, do not accumulate on the fabric after repeated laundry washing.

The invention further provides a process for producing such a novel cellulase.

In another aspect, the invention provides detergent compositions, detergent additives, fabric softeners and depilling compositions comprising the novel cellulase.

Still another aspect of the invention is the use of the novel cellulases in methods for treating cotton containing textiles, like 'Stone wash' and 'Biopolish' processes.

#### Legend to the figure

Figure 1 shows the relative activities of the cellulase obtainable from E. coli clone BCE 103. In Example 3 this figure is referred to as the pH/temperature profiles. All activities for both 40 and 60°C are related to the highest activity which is fixed on 100%.

Detailed disclosure of the invention

As noted above, the present invention generally relates to a novel cellulase and its applications. However, prior to disclosing this invention in detail, first the following terms will be defined.

"Cellulase" is a generic name for enzymes acting on cellulose and its derivatives, and hydrolysing them into glucose, cellobiose or cellooligosaccharides.

The term "single" cellulase used herein is intended to mean a cellulase which is produced by one gene.

The term "cleaning" means the removal of dirt attached to laundry.

The term "pilling" in this respect is the formation of pills and fuzz on the surface of cotton containing fabrics due to broken or disordered fibres.

The term "depilling" is the removal of pills and fuzz from cotton containing fabrics. Depilling results in colour clarification when coloured cotton containing fabrics are depilled.

The term "colour clarification" in this respect is the reestablishment of the attractive fresh look of coloured fabrics containing or consisting of cellulose based fibres, which have developed a greyish appearance by a cellulase treatment of the coloured fabric.

The term "antiredeposition" in this respect is the action of cellulase to prevent or diminish the redeposition of dirt and colour components on the fabric.

The term "redeposition" in this respect is deposition of dirt or colour components that were removed from these textiles or fabrics during a laundry washing or textile treatment.

The term "derivative" is intended to indicate a protein which is derived from the native protein by addition of one or more amino acids to either or both the C- and N-terminal end of the native protein, substitution of one or more amino acids at one or a number of different sites in the native amino acid sequence, deletion of one or more

amino acids at either or both ends of the native protein or at one or more sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence.

5

The present invention relates to a novel cellulase which is obtainable from the following microorganism which is deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms  
10 for the Purposes of Patent Procedures, at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands on December 23, 1993 under deposition number CBS 670.93 (already described in copending application PCT/EP94/04312). This strain is classified as a new species of the genus  
15 Bacillus, which does not belong to any of the presently known rRNA-groups of Bacillus.

The microorganism may be obtained for example from water and soil samples collected in alkaline environments such as alkaline soils and soda lakes.

20 The microorganisms have subsequently been screened using a carboxymethyl cellulose (CMC)-agar diffusion assay. Strains which showed a clearing zone in this test were isolated as potential cellulase producing strains. Genomic gene libraries of the alkali tolerant cellulase producing  
25 strains were constructed. Recombinant clones were screened by agar diffusion on CMC-agar. Recombinant clones that showed clearing zones around the colony were isolated. Single cellulases were produced by fermentation of the recombinant clones in 4\*YEP-medium for 48 hours at 30°C. The  
30 obtained single cellulases optionally purified as described in Example 2 were tested in the following tests:

- a) Anti redeposition test;
- b) Depilling test.

Surprisingly we have found that the cellulase obtainable  
35 from CBS 670.93 shows a good performance in both tests.

The present invention discloses a cellulase which cellulase exhibit the following properties:

(a) show a delta REM of at least 4 units in the Anti Redeposition Test and

(b) show a depilling result which depilling result is at least comparable to that of the cellulase obtainable  
5 from Bacillus sp. CBS 670.93 in the Depilling Test.

The Anti Redeposition Test is described in Example 4. Whiteness maintenance of white fabric is measured by a reflectance measurement. The higher the reflectance value, the more effective is the tested cellulase in  
10 antiredeposition performance.

The Depilling Test is described in Example 5. Depilling is the removal of fibres that are disordered and/or broken which make the coloured cotton containing fabric look greyish. The more disordered and/or broken  
15 fibres are removed the better the coloured cotton containing fabrics look. Depilling effectiveness can be judged by panels or can be quantified by an image analysis system. In the image analysis system an area of 4x4 cm is transferred from a surface microscope to a CCD camera connected to an  
20 image analyzing system. The pilling is indicated as a percentage of white area on the dark area of unpilled textile. The results can be given as a percentage of pilled area.

The cellulase of the present invention was further  
25 characterized by the Fibre Damage test. This test is described in Example 4. Surprisingly it has been found that the cellulase of the invention, while showing a good depilling effect does not show much fibre damage. The fibre damage can further be quantified by a tensile strength test,  
30 as described in International Standard ISO 2267.

The cellulase of the present invention was even further characterized by the Adsorption test. This test is described in Example 4. Low adsorption of the cellulase to the cotton is desired. Surprisingly it has been found that  
35 the cellulase of the invention, while showing a good depilling effect do not adsorb to the cotton as much as previously known depilling cellulases do.



Results from small scale experiments can be confirmed by full scale laundry washing experiments. The cellulase of the present invention can further be characterized by the pH and temperature activity profiles. These profiles can be made by using the CMC'ase assay as described in Example 3. By varying the pH or temperature at the enzyme incubation, pH and temperature profiles can be obtained. For determination of the pH profile a phosphate/citrate buffer system can be used.

10 The present invention also discloses a process for the production of the cellulase of the present invention, which can be developed using genetic engineering. As a first step the gene encoding the cellulase of the present invention can be cloned using  $\lambda$ -phage (expression) vectors and E. coli host cells. (Alternatively PCR cloning using consensus primers designed on conserved domains may be used.) Expression of the gene encoding the cellulase of the present invention in E. coli, is shown to give an active protein.

20 After a first cloning step in E. coli, a cellulase gene can be transferred to a more preferred industrial expression host such as Bacillus or Streptomyces species, a filamentous fungus such as Aspergillus, or a yeast. High level expression and secretion obtainable in these host organisms allows accumulation of the cellulase of the invention in the fermentation medium from which they can subsequently be recovered.

The present invention further relates to a detergent composition which comprises the above described cellulase.

30 Detergent compositions comprising the inventive cellulase may additionally comprise surfactants which may be of the anionic, non-ionic, cationic, amphoteric or zwitterionic type as well as mixtures of these surfactant classes. Examples of surfactants are described in GB 2094826-A (Kao Corporation).

Detergent compositions of the invention may contain other detergent ingredients known in the art as e.g.

builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, enzyme stabilizers, etc.

The detergent compositions of the invention may be formulated in any convenient form e.g. as a powder or liquid.

Fabric softening compositions comprising the inventive cellulase may further comprise cationic surfactants which are capable of fabric softening.

Depilling compositions comprising the inventive cellulase may further comprise surfactants and/or pH stabilizers. Depilling compositions can be used for a one time treatment of cotton containing fabrics.

The present invention further relates to the use of the above described cellulase in wet processing of cellulosic-based fabrics or garments.

For example cellulases are used to give an abraded look to ring dyed cellulosic goods, in particular to achieve variations in colour density in indigo-dyed denim products providing the popular stonewashed look of denim jeans (described in EP 307564). The cellulases replace or reduce the amount of pumice stones that used to be applied in jeans manufacturing.

Another example is the "Biopolish" process (described by Asferg et al. (1990), Int. Textile Bulletin, Dyeing/Printing/Finishing, 36, 5-8) in which cellulases are applied to improve the quality of cellulosic fabrics. Typical biopolish effects are for example a better feel & handling, decreased amount of fuzz, lower tendency to form pills (pilling prevention), improved softness or increased luster in comparison to the untreated fabric.

It has been found that the cellulase of the present invention is capable of both providing a Stone Washed effect and a Biopolish effect when used in these processes.

The invention will be explained in more detail in the following examples which are provided for illustration and are not to be construed as limiting on the invention.

### Example 1

#### **Screening for cellulase producing microorganisms**

Two methods were applied for the isolation of cellulase-producing microorganisms:

5 i) the soil and water samples were suspended in 0.85% saline solution and directly used in the carboxymethyl cellulose (CMC)-agar diffusion assay for detection of cellulase producing colonies.

10 ii) The soil and water samples were enriched for cellulase containing strains by incubation in a cellulose containing liquid minimal medium or GAM-medium for 1 to 3 days at 40°C. Cultures that showed bacterial growth were analyzed for cellulase activity using the CMC-agar diffusion assay for detection of cellulase producing colonies.

15

#### **Isolation of alkalitolerant, cellulase producing strains**

Strains that showed clearing zones in the agar diffusion assay were fermented in 25 millilitre GAM-medium in 100 millilitre shake flasks in an Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA), at 250 r.p.m. at 20 40°C for 72 hours. CMCase activity was determined in the culture broth at pH 9 and 40°C.

#### **Isolation of cellulase genes**

25 Genomic gene libraries of the alkalitolerant cellulase producing strains were constructed in plasmid pTZ18R (Mead, D.A., et al. (1986) Protein Engineering 1, 67). Recombinant clones were screened by agar diffusion on CMC-agar as described by Wood, P.J., et al. (1988) Methods 30 in Enzymology 160, 59-74. Strains that showed clearing zones around the colony were isolated. The CMCase activity of the recombinant strains was determined after fermentation for 48 hours at 30°C in 4\*YEP-medium. The plasmid DNA of the recombinant strains was isolated and the inserts were 35 characterized by restriction enzyme analysis and nucleotide sequence analysis.

## Media

The minimal medium (pH 9.7) used in the CMC-agar diffusion assay and the enrichment procedure, consisted of KNO<sub>3</sub> 1%, Yeast extract (Difco) 0.1%, KH<sub>2</sub>PO<sub>4</sub> 0.1%, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.02%, Na<sub>2</sub>CO<sub>3</sub> 1%, NaCl 4% and 0.25% CMC (Sigma C-4888). For solidification 1.5% agar was added.

The complex medium (GAM) used for enzyme production of the donor strains consisted of Peptone (Difco) 0.5%, Yeast extract (Difco) 0.5%, Glucose.H<sub>2</sub>O 1%, KH<sub>2</sub>PO<sub>4</sub> 0.1%, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.02%, Na<sub>2</sub>CO<sub>3</sub> 1%, NaCl 4%. The pH was adjusted to 9.5 with 4M HCl after which 1% CMC was added.

The complex medium (4\*YEP) used for the enzyme production in E. coli recombinant strains consisted of Yeast extract (Difco) 4%, Peptone (Difco) 8%, lactose 0.2%, 100 µg/ml ampicilline).

## CMC-agar diffusion assay for colonies

Cell suspensions in 0.85% saline solution were plated on CMC-containing minimal medium. After incubation for 1 to 3 days at 40°C, the plates were replica plated and the parent plate was flooded with 0.1% Congo Red for 15 minutes. The plates were destained with 1M NaCl for 30 minutes. The strains that showed a clearing zone around the colony were isolated as potential cellulases producing microorganisms.

## CMC-agar diffusion assay for liquid fractions

Aliquots of 40 µl of enzyme solution or fermentation broth were pipetted in wells punched out from a layer of 5 mm of minimal medium in a petri dish. After incubation for 16 hours at 40°C cellulase activity was detected by Congo Red / NaCl treatment. The diameter of the clearing zone is a measure for the CMCase activity.

## Results of Example 1

The experiments of Example 1 resulted in the isolation of a cellulase producing microorganism which was

deposited thereafter as CBS 670.93. The microorganism was classified as a new species of the genus Bacillus. Cloning experiments of Example 1 with the CBS 670.93 strain as a donor strain resulted in the isolation of an E. coli clone  
5 called BCE 103 which was able to produce a cellulase. The nucleotide sequence of the gene coding for said cellulase was analysed. From the cellulase produced by BCE 103 the N-terminal amino acid sequence was determined using standard methods for obtaining and sequencing peptides (Finlay &  
10 Geisow (Eds.), Protein Sequencing - a practical approach, 1989, IRL Press). The amino acid sequence of the cellulase was deduced from the nucleotide sequence, using the N-terminal amino acid sequence for the starting point of the mature protein.

15 The nucleotide sequence is shown in SEQ ID No. 1 and the amino acid sequence is shown in SEQ ID No. 2.

## Example 2

### 20 Purification of cellulases

After the fermentation the cells were separated from the culture liquid by centrifugation (8000 rpm). The cellulase in the supernatant was precipitated with ammonium sulphate (65% saturation). The precipitate was dissolved in  
25 25 mM phosphate buffer pH 7 + 5 mM EDTA until a conductivity of 7 mS/cm. This solution was applied to a Q-Sepharose FF (diameter 5 cm, length 10 cm) Anion Exchange column, after which the column was washed with 25 mM phosphate buffer pH 7 + 5 mM EDTA until an absorbency of 0.2 AU. A gradient of 0  
30 to 0.5 M NaCl in 25 mM phosphate pH 7 was applied to the column in 80 minutes followed by a gradient from 0.5 to 1 M NaCl in 10 minutes. Depending on which cellulase was applied to the column, elution took place in the first or the second gradient. After elution the column was cleaned (upflow) with  
35 1 M NaOH and equilibrated again with 25 mM phosphate pH 7 + 5 mM EDTA.

Depending on the elution the obtained cellulase had a purity of up to about 80%.

### Example 3

#### 5 Characterization of cellulases

##### CMC'ase assay

Assays for cellulase activity were performed using modified methods of the PAHBAH method (Lever M. Anal. Biochem. 1972, 47, 273-279 and Lever M. Anal. Biochem. 1977, 10 81, 21-27).

##### Procedure

A test tube is filled with 250  $\mu$ l 2.5% CMC in 50 mM glycine buffer pH 9 (CMC-low viscosity is purchased from 15 Sigma) and 250  $\mu$ l aliquots cellulase, diluted in the appropriate buffer. The test tube is incubated for 30 minutes at 40°C in a waterbath, whereafter 1.5 ml of a daily fresh prepared PAHBAH solution (1% PAHBAH in 100 ml 0.5 M NaOH with 100  $\mu$ l bismuth solution (containing 48.5 g bismuth 20 nitrate, 28.2 g potassium sodium tartrate and 12.0 g NaOH in 100 ml) is added. The mixture is heated at 70°C for 10 minutes, after which it is cooled on ice for 2 minutes. The absorption is measured at 410 nm. To eliminate the background absorbance of the enzyme samples a control 25 experiment is executed as follows: a tube with substrate is incubated under the same conditions as the test tube. After the incubation 1.5 ml PAHBAH and the enzyme preparation is added (in this order). One unit (U) is defined as the amount of enzyme producing 1  $\mu$ mol of glucose from CMC equivalent 30 determined as reducing sugars per minute per gram product.

The buffer used for the determination of the pH/temperature profiles is a phosphate/citrate system. The pH/temperature profiles were determined using a fixed enzyme concentration which fits in the linear range of the dose 35 response profile measured at pH 7 and 40°C. This enzyme concentration was used for the measurement of the activities under all other determined conditions.

The results for the cellulase produced by the BCE 103 clone are shown in Figure I. The cellulase produced by the BCE 103 clone shows good activities at alkaline pH, which makes it suitable for application in detergents with an alkaline pH.

#### Example 4

##### **Anti redeposition test**

##### Procedure

20 ml 0.5% pigmented soil (fresh prepared, daily and consisting of 86% kaolin, 8% soot (from Degussa Flamm-ruß101), 4% iron oxide black and 2% iron oxide yellow (from Henkel Genthin GmbH)), in a detergent (Persil color without enzymes, 5 g/l, pH 8.5) was, under agitating (90 rpm) incubated with white cotton fabric (Windelbleiche, Krefeld, prewashed 5 cm diameter). Cellulase was added until a final concentration of 1 mU/ml. The mixture was incubated for 30 minutes at 40°C, 90 rpm. As a control the same incubation was carried out without the addition of cellulase. After the incubation the fabric was rinsed thoroughly with running cold water.

After drying the whiteness of the fabric was measured by remission (4 measurements per fabric) using a Micro color Dr. Lange Colorimeter. The control value was subtracted from the sample value.

The results, expressed as delta Rem, are shown in Table 1.

##### **Fibre Damage Test**

##### Procedure:

One pad of cotton wool ('Wattenpads', 100% cotton, Warenhandels GmbH, Buchholz, Marke Olivia, Selling agency: Aldi) was incubated in 40 ml wash liquor (Persil color, without enzyme, 5 g/l pH 8.5), cellulase at a final concentration of 1 mU/ml was added in a sealed flask and incubated for 20 hours at 40°C under agitation (90 rpm). After the incubation, fibre damage was monitored by the



measurement of the quantity of the reducing sugars in solution, using the PAHBAH method described in Example 3. As a control the same incubation was carried out without the addition of cellulase.

5 The results are shown in Table 1.

### Adsorption Test

#### Procedure:

White cotton fabric (Windelbleiche, Bielefeld)  
10 prewashed with Persil without enzymes at 60°C, was cut round to 9 cm diameter (approx. 0.920 gram). One cotton swatch was incubated in 50 ml 50 mM glycine-NaOH buffer pH 9 including 0.1% SDS and 1 ml cellulase sample (600 mU/ml) for 60 minutes at 30°C. 2 ml samples were taken at T=0 and at T=60  
15 minutes and were diluted directly (1:2) with 50 mM MES-buffer pH 6.5 and stored at 4°C until measurement. As control the same incubation was carried out without the addition of cotton textile. The activity measurement was determined with a PAHBAH method as described in Example 3,  
20 but at pH 6.5 in 50 mM MES buffer.

The adsorption was expressed as relative adsorption where the activity applied at the start of the experiment was set as 100%, T=0. 100% activity value - remaining activity (%) = adsorption (%).

25 The results are shown in Table 1.

Table 1: results of the Antiredeposition Test, Fibre Damage Test and Adsorption Test

Enzyme	Antiredeposition (delta REM)	Fibre Damage (mU)	Adsorption (%)
30 BCE 103	5.0	0.025	7
Kao Kac® *	7.5	0.006	0
Denimax Ultra®MG **	1.2	0.155	36

\* Kao Kac® = cellulase of Kao Corporation, capable in cleaning performance but not in depilling performance.

35 \*\* Denimax Ultra®MG = cellulase of Novo/Nordisk, capable in

depilling performance but not in cleaning performance.

Example 5

5 Depilling test

Depilling and colour clarification performance were determined by treating pilled worn cotton fabric several times with cellulase. After washing the fabric was judged based on depilling and colour clarification compared to a control fabric which had not been treated with the enzyme.

One wash cycle consisted of the following steps:  
Four pilled and dark coloured cotton swatches (4x4 cm) were incubated in 40 ml wash liquor (5 g/l All color, pH 8.6) in a glass beaker (150 ml). The wash was performed at 40°C for 30 minutes in a shaken water bath (maximum shaking). After the incubation the fabrics were rinsed thoroughly for 10 minutes with running tap water and dried in a tumble dryer. Cellulase dosage is 2.5 mg/ml protein (BCA Pierce, BSA as standard).

20 As a control the same wash cycle was carried out without the addition of cellulase.

A total of 20 washing cycles were carried out. After every 5 wash cycles one fabric was taken out and a new one was added to the beaker in order to maintain the same fabric liquor ratio. After 20 washing cycles the fabrics were evaluated (on visual appearance) with the use of a panel, on a scale of 1 to 4, compared to the control, whereas:

- 1 = no depilling/colour clarification
- 2 = slight depilling/colour clarification
- 3 = good depilling/colour clarification
- 4 = very good depilling/colour clarification

The results are summarized in Table 3.

Table 2: Results of the Depilling Test

Sample	Colour clarification, depilling
BCE 103	4
Kao Kac <sup>®</sup> *	1
Denimax Ultra <sup>®</sup> MG **	4

\*Kao Kac<sup>®</sup> = as described in Example 4

\*\* Denimax Ultra<sup>®</sup>MG = as described in Example 4

10 Example 6

**Desizing**

A UniMac<sup>®</sup> Rotary Washer/Extractor-model UY 230 machine was loaded with 10 pounds of Swift Denim style #27261 (rigid, 100% cotton indigo dyed 14+ ounce per square yard denim). The Unimac was filled with hot water to a liquor ratio of 20:1 and heated to 82°C under slow rotation (5 rpm). 1.0 gram/liter Rapidstrip<sup>®</sup>102 (amylase) was added to the machine and the garments were allowed to soak for 3 minutes without rotation. Desizing took place for 15 minutes at 40 rpm.

After desizing the machine was drained and again filled with hot water to a liquor ratio of 20:1 and heated to 60°C under a low rotation (5 rpm). The garments were rinsed for 2 minutes after which the machine was drained. Again the machine was filled with hot water to a liquor ratio of 20:1 and heated to 55°C under low rotation (5 rpm). The garments were rinsed for 2 minutes after which the machine was drained.

**Stonewash**

Directly after the desize procedure the machine was filled to a liquor ratio of 6:1 (30 liters) and was heated to a temperature desired for cellulase abrasion. 0.1 M K<sub>2</sub>HPO<sub>4</sub> was added to the desired pH for abrasion. 72 mg Cellulase (BCE 103) was added to the machine and the garments were

abraded for 60 minutes at 33 rpm, after which the machine was drained. After draining the machine was filled with hot water to a liquor ratio of 20:1 and heated to 60°C under a slow rotation (5 rpm). The garments were rinsed for 2 minutes after which the machine was drained. Again the machine was filled with hot water to a liquor ratio of 20:1 and heated to 37°C under a slow rotation (5 rpm). The garments were rinsed for 2 minutes after which the machine was drained and the garments tumbled dry.

The tested conditions were pH 7, 40°C and pH 7.5, 60°C. The results are shown in Table 3.

The jeans were evaluated (on visual appearance) on abrasion and indigo redeposition with the use of a panel, whereas for abrasion:

- 1] no abrasion
- 2] significant abrasion

for indigo redeposition:

- 1] acceptable level of indigo redeposition
- 2] unacceptable level of indigo redeposition

Table 3

Results of stonewash test

Sample	dosage	pH	Temp	Abrasion	indigo redeposition
BCE 103	72 mg	7.0	40°C	2	1
BCE 103	72 mg	7.5	60°C	2	1
No enzyme	-	7.0	40°C	1	-

GAG Glu	GAA Glu	CAT His	GGG Gly 10	CAA Gln	CTA Leu	AGT Ser	ATT Ile	AGT Ser 15	AAC Asn	GGT Gly	GAA Glu	TTA Leu	GTC Val 20	AAT Asn	GAA Glu	144
CGA Arg	GGC Gly	GAA Glu 25	CAA Gln	GTT Val	CAG Gln	TTA Leu	AAA Lys 30	GGG Gly	ATG Met	AGT Ser	TCC Ser	CAT His 35	GGT Gly	TTG Leu	CAA Gln	192
TGG Trp	TAC Tyr 40	GGT Gly	CAA Gln	TTT Phe	GTA Val	AAC Asn 45	TAT Tyr	GAA Glu	AGC Ser	ATG Met	AAA Lys 50	TGG Trp	CTA Leu	AGA Arg	GAT Asp	240
GAT Asp 55	TGG Trp	GGA Gly	ATA Ile	ACT Thr	GTA Val 60	TTC Phe	CGA Arg	GCA Ala	GCA Ala	ATG Met 65	TAT Tyr	ACC Thr	TCT Ser	TCA Ser	GGA Gly 70	288
GGA Gly	TAT Tyr	ATT Ile	GAC Asp 75	GAT Asp	CCA Pro	TCA Ser	GTA Val	AAG Lys 80	GAA Glu	AAA Lys	GTA Val	AAA Lys	GAG Glu	ACT Thr 85	GTT Val	336
GAG Glu	GCT Ala	GCG Ala	ATA Ile 90	GAC Asp	CTT Leu	GGC Gly	ATA Ile 95	TAT Tyr	GTG Val	ATC Ile	ATT Ile	GAT Asp	TGG Trp 100	CAT His	ATC Ile	384
CTT Leu	TCA Ser	GAC Asp 105	AAT Asn	GAC Asp	CCG Pro	AAT Asn 110	ATA Ile	TAT Tyr	AAA Lys	GAA Glu	GAA Glu	GCG Ala 115	AAG Lys	GAT Asp	TTC Phe	432
TTT Phe 120	GAT Asp	GAA Glu	ATG Met	TCA Ser	GAG Glu 125	TTG Leu	TAT Tyr	GGA Gly	GAC Asp	TAT Tyr	CCG Pro 130	AAT Asn	GTG Val	ATA Ile	TAC Tyr	480
GAA Glu 135	ATT Ile	GCA Ala	AAT Asn	GAA Glu	CCG Pro 140	AAT Asn	GGT Gly	AGT Ser	GAT Asp	GTT Val 145	ACG Thr	TGG Trp	GAC Asp	AAT Asn	CAA Gln 150	528
ATA Ile	AAA Lys	CCG Pro	TAT Tyr 155	GCA Ala	GAA Glu	GAA Glu	GTG Val	ATT Ile	CCG Pro 160	GTT Val	ATT Ile	CGT Arg	GAC Asp 165	AAT Asn	GAC Asp	576
CCT Pro	AAT Asn	AAC Asn	ATT Ile 170	GTT Val	ATT Ile	GTA Val	GGT Gly 175	ACA Thr	GGT Gly	ACA Thr	TGG Trp	AGT Ser	CAG Gln 180	GAT Asp	GTC Val	624
CAT His	CAT His	GCA Ala 185	GCC Ala	GAT Asp	AAT Asn	CAG Gln	CTT Leu 190	GCA Ala	GAT Asp	CCT Pro	AAC Asn	GTC Val 195	ATG Met	TAT Tyr	GCA Ala	672
TTT Phe 200	CAT His	TTT Phe	TAT Tyr	GCA Ala	GGA Gly	ACA Thr 205	CAT His	GGA Gly	CAA Gln	AAT Asn	TTA Leu 210	CGA Arg	GAC Asp	CAA Gln	GTA Val	720
GAT Asp 215	TAT Tyr	GCA Ala	TTA Leu	GAT Asp	CAA Gln 220	GGA Gly	GCA Ala	GCG Ala	ATA Ile	TTT Phe 225	GTT Val	AGT Ser	GAA Glu	TGG Trp	GGG Gly 230	768
ACA Thr	AGT Ser	GCA Ala	GCT Ala 235	ACA Thr	GGT Gly	GAT Asp	GGT Gly	GGT Gly	GTG Val 240	TTT Phe	TTA Leu	GAT Asp	GAA Glu	GCA Ala 245	CAA Gln	816
GTG Val	TGG Trp	ATT Ile 250	GAC Asp	TTT Phe	ATG Met	GAT Asp	GAA Glu	AGA Arg 255	AAT Asn	TTA Leu	AGC Ser	TGG Trp	GCC Ala 260	AAC Asn	TGG Trp	864
TCT Ser	CTA Leu	ACG Thr 265	CAT His	AAG Lys	GAT Asp	GAG Glu	TCA Ser 270	TCT Ser	GCA Ala	GCG Ala	TTA Leu	ATG Met 275	CCA Pro	GGT Gly	GCA Ala	912

# SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT:
  - (A) NAME: Gist-brocades
  - (B) STREET: Wateringseweg 1
  - (C) CITY: Delft
  - (E) COUNTRY: The Netherlands
  - (F) POSTAL CODE (ZIP): 2611 XT
- (ii) TITLE OF INVENTION: Novel Cellulase and Its Applications
- (iii) NUMBER OF SEQUENCES: 2
- (iv) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1404 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Bacillus sp.
  - (C) INDIVIDUAL ISOLATE: CBS 670.93
- (ix) FEATURE:
  - (A) NAME/KEY: sig\_peptide
  - (B) LOCATION: 1..78
- (ix) FEATURE:
  - (A) NAME/KEY: mat\_peptide
  - (B) LOCATION: 79..1404
  - (D) OTHER INFORMATION: /function= "endoglucanase"  
/EC\_number= 3.2.1.4  
/product= "BCE103 cellulase"
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 1..1404

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG AAA AAG ATA ACT ACT ATT TTT GCC GTA TTG CTC ATG ACA TTG GCG	48
Met Lys Lys Ile Thr Thr Ile Phe Ala Val Leu Leu Met Thr Leu Ala	
-26 -25 -20 -15	
TTG TTC AGT ATA GGA AAC ACG ACA GCG GCT GAT GAT TAT TCA GTT GTA	96
Leu Phe Ser Ile Gly Asn Thr Thr Ala Ala Asp Asp Tyr Ser Val Val	
-10 -5 1 5	

AAT CCA ACT GGT GGT TGG ACA GAG GCT GAA CTA TCT CCA TCT GGT ACA	960
Asn Pro Thr Gly Gly Trp Thr Glu Ala Glu Leu Ser Pro Ser Gly Thr	
280 285 290	
TTT GTG AGG GAA AAA ATA AGA GAA TCA GCA TCT ATT CCG CCA AGC GAT	1008
Phe Val Arg Glu Lys Ile Arg Glu Ser Ala Ser Ile Pro Pro Ser Asp	
295 300 305 310	
CCA ACA CCG CCA TCT GAT CCA GGA GAA CCG GAT CCA GGA GAA CCG GAT	1056
Pro Thr Pro Pro Ser Asp Pro Gly Glu Pro Asp Pro Gly Glu Pro Asp	
315 320 325	
CCA ACG CCC CCA AGT GAT CCA GGA GAG TAT CCA GCA TGG GAT TCA AAT	1104
Pro Thr Pro Pro Ser Asp Pro Gly Glu Tyr Pro Ala Trp Asp Ser Asn	
330 335 340	
CAA ATT TAC ACA AAT GAA ATT GTG TAT CAT AAC GGT CAG TTA TGG CAA	1152
Gln Ile Tyr Thr Asn Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln	
345 350 355	
GCG AAA TGG TGG ACA CAA AAT CAA GAG CCA GGT GAC CCA TAC GGT CCG	1200
Ala Lys Trp Trp Thr Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro	
360 365 370	
TGG GAA CCA CTC AAA TCT GAC CCA GAT TCA GGA GAA CCG GAT CCA ACG	1248
Trp Glu Pro Leu Lys Ser Asp Pro Asp Ser Gly Glu Pro Asp Pro Thr	
375 380 385 390	
CCC CCA AGT GAT CCA GGA GAG TAT CCA GCA TGG GAT TCA AAT CAA ATT	1296
Pro Pro Ser Asp Pro Gly Glu Tyr Pro Ala Trp Asp Ser Asn Gln Ile	
395 400 405	
TAC ACA AAT GAA ATT GTG TAC CAT AAC GGC CAG CTA TGG CAA GCA AAA	1344
Tyr Thr Asn Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys	
410 415 420	
TGG TGG ACA CAA AAT CAA GAG CCA GGT GAC CCA TAT GGT CCG TGG GAA	1392
Trp Trp Thr Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu	
425 430 435	
CCA CTC AAT TAA	1404
Pro Leu Asn	
440	

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 467 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Lys Ile Thr Thr Ile Phe Ala Val Leu Leu Met Thr Leu Ala	
-26 -25 -20 -15	
Leu Phe Ser Ile Gly Asn Thr Thr Ala Ala Asp Asp Tyr Ser Val Val	
-10 -5 1 5	
Glu Glu His Gly Gln Leu Ser Ile Ser Asn Gly Glu Leu Val Asn Glu	
10 15 20	
Arg Gly Glu Gln Val Gln Leu Lys Gly Met Ser Ser His Gly Leu Gln	
25 30 35	

Trp	Tyr	Gly	Gln	Phe	Val	Asn	Tyr	Glu	Ser	Met	Lys	Trp	Leu	Arg	Asp
	40					45					50				
Asp	Trp	Gly	Ile	Thr	Val	Phe	Arg	Ala	Ala	Met	Tyr	Thr	Ser	Ser	Gly
55					60					65					70
Gly	Tyr	Ile	Asp	Asp	Pro	Ser	Val	Lys	Glu	Lys	Val	Lys	Glu	Thr	Val
			75					80						85	
Glu	Ala	Ala	Ile	Asp	Leu	Gly	Ile	Tyr	Val	Ile	Ile	Asp	Trp	His	Ile
			90					95					100		
Leu	Ser	Asp	Asn	Asp	Pro	Asn	Ile	Tyr	Lys	Glu	Glu	Ala	Lys	Asp	Phe
		105					110					115			
Phe	Asp	Glu	Met	Ser	Glu	Leu	Tyr	Gly	Asp	Tyr	Pro	Asn	Val	Ile	Tyr
	120					125					130				
Glu	Ile	Ala	Asn	Glu	Pro	Asn	Gly	Ser	Asp	Val	Thr	Trp	Asp	Asn	Gln
135					140					145					150
Ile	Lys	Pro	Tyr	Ala	Glu	Glu	Val	Ile	Pro	Val	Ile	Arg	Asp	Asn	Asp
				155					160					165	
Pro	Asn	Asn	Ile	Val	Ile	Val	Gly	Thr	Gly	Thr	Trp	Ser	Gln	Asp	Val
			170					175					180		
His	His	Ala	Ala	Asp	Asn	Gln	Leu	Ala	Asp	Pro	Asn	Val	Met	Tyr	Ala
		185					190					195			
Phe	His	Phe	Tyr	Ala	Gly	Thr	His	Gly	Gln	Asn	Leu	Arg	Asp	Gln	Val
	200					205					210				
Asp	Tyr	Ala	Leu	Asp	Gln	Gly	Ala	Ala	Ile	Phe	Val	Ser	Glu	Trp	Gly
215					220					225					230
Thr	Ser	Ala	Ala	Thr	Gly	Asp	Gly	Gly	Val	Phe	Leu	Asp	Glu	Ala	Gln
				235					240					245	
Val	Trp	Ile	Asp	Phe	Met	Asp	Glu	Arg	Asn	Leu	Ser	Trp	Ala	Asn	Trp
			250					255					260		
Ser	Leu	Thr	His	Lys	Asp	Glu	Ser	Ser	Ala	Ala	Leu	Met	Pro	Gly	Ala
		265					270					275			
Asn	Pro	Thr	Gly	Gly	Trp	Thr	Glu	Ala	Glu	Leu	Ser	Pro	Ser	Gly	Thr
	280					285					290				
Phe	Val	Arg	Glu	Lys	Ile	Arg	Glu	Ser	Ala	Ser	Ile	Pro	Pro	Ser	Asp
295					300					305					310
Pro	Thr	Pro	Pro	Ser	Asp	Pro	Gly	Glu	Pro	Asp	Pro	Gly	Glu	Pro	Asp
				315					320					325	
Pro	Thr	Pro	Pro	Ser	Asp	Pro	Gly	Glu	Tyr	Pro	Ala	Trp	Asp	Ser	Asn
			330					335					340		
Gln	Ile	Tyr	Thr	Asn	Glu	Ile	Val	Tyr	His	Asn	Gly	Gln	Leu	Trp	Gln
		345					350					355			
Ala	Lys	Trp	Trp	Thr	Gln	Asn	Gln	Glu	Pro	Gly	Asp	Pro	Tyr	Gly	Pro
	360					365					370				
Trp	Glu	Pro	Leu	Lys	Ser	Asp	Pro	Asp	Ser	Gly	Glu	Pro	Asp	Pro	Thr
375					380					385					390



Pro Pro Ser Asp Pro Gly Glu Tyr Pro Ala Trp Asp Ser Asn Gln Ile  
395 400 405

Tyr Thr Asn Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys  
410 415 420

Trp Trp Thr Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu  
425 430 435

Pro Leu Asn  
440

Claims

1. Cellulase which exhibit the following properties:
- 5 (a) show a delta REM of at least 4 units, preferably at least 5 units, in the Anti Redeposition Test, and
- (b) show a depilling result which depilling result is at least comparable to that of the cellulase obtainable from CBS 670.93 in the Depilling Test.
- 10
2. A cellulase according to claim 1 which cellulase further exhibits
- (c) a fibre damage of less than 0.05 milliunits in
- 15 the Fibre Damage Test.
3. A cellulase according to any one of claims 1 or 2, which cellulase further exhibits
- (d) an adsorption of less than 15% in the Adsorption
- 20 Test.
4. A cellulase according to any one of claims 1 to 3, which is obtainable from a microorganism, preferably a fungus or a bacterium.
- 25
5. A cellulase according to claim 4 wherein the bacterium belongs to the genus Bacillus, preferably an alkalophilic Bacillus, more preferably Bacillus sp. CBS 670.93.
- 30
6. A cellulase according to any one of claims 1 to 5, wherein the cellulase has the amino acid sequence as listed in SEQ ID No. 2 or a derivative thereof.
- 35
7. An isolated DNA sequence encoding a cellulase according to any one of claims 1-6.

8. A vector capable of transforming a microbial host cell and characterized in that the vector comprises a DNA sequence according to claim 7.

5           9. A vector according to claim 8 and characterized in that the DNA sequence is operably linked to expression signals that ensure the expression of the DNA sequence in the microbial host.

10           10. A microbial host which contains a vector according to claims 8 or 9.

15           11. A microbial host according to claim 10 and characterized in that the microbial host expresses the DNA sequence.

12. A process for the preparation of the cellulase according to any one of claims 1 to 6, and characterized in that a microorganism producing the cellulase is cultivated  
20 in a suitable medium, whereafter the produced cellulase is recovered.

13. A detergent composition which comprises a cellulase according to any one of claims 1 to 6.

25

14. A detergent composition according to claim 13, wherein the detergent composition may be a granular or liquid detergent.

30

15. A detergent composition according to claim 13 or 14, wherein the detergent composition further comprises a surfactant and a builder.

16. The use of a detergent composition according to  
35 any one of claims 13 to 15.

17. The use of a cellulase according to any one of claims 1 to 6, in a textile treatment.

18. The use of a cellulase according to claim 17,  
s wherein the textile treatment is a stone wash process or a biopolish process.

19. A fabric softener composition which comprises a cellulase according to any one of claims 1 to 6.

10

20. A fabric softener composition according to claim 19 wherein the fabric softener composition further comprises a cationic surfactant capable of providing fabric softening.

15

21. A depilling treatment composition which comprises a cellulase according to any one of claims 1 to 6.

ABSTRACT

The present invention discloses an enzyme having cellulase activity. The (single cellulase is characterized in that it is able to provide both antiredeposition and depilling effects when applied in laundry washing. The enzyme is obtainable from a deposited strain which is classified as a new species of the genus Bacillus. The enzyme is suited for use in detergent- and textile-treatment applications.

11/4  
6/

relative activity (%)

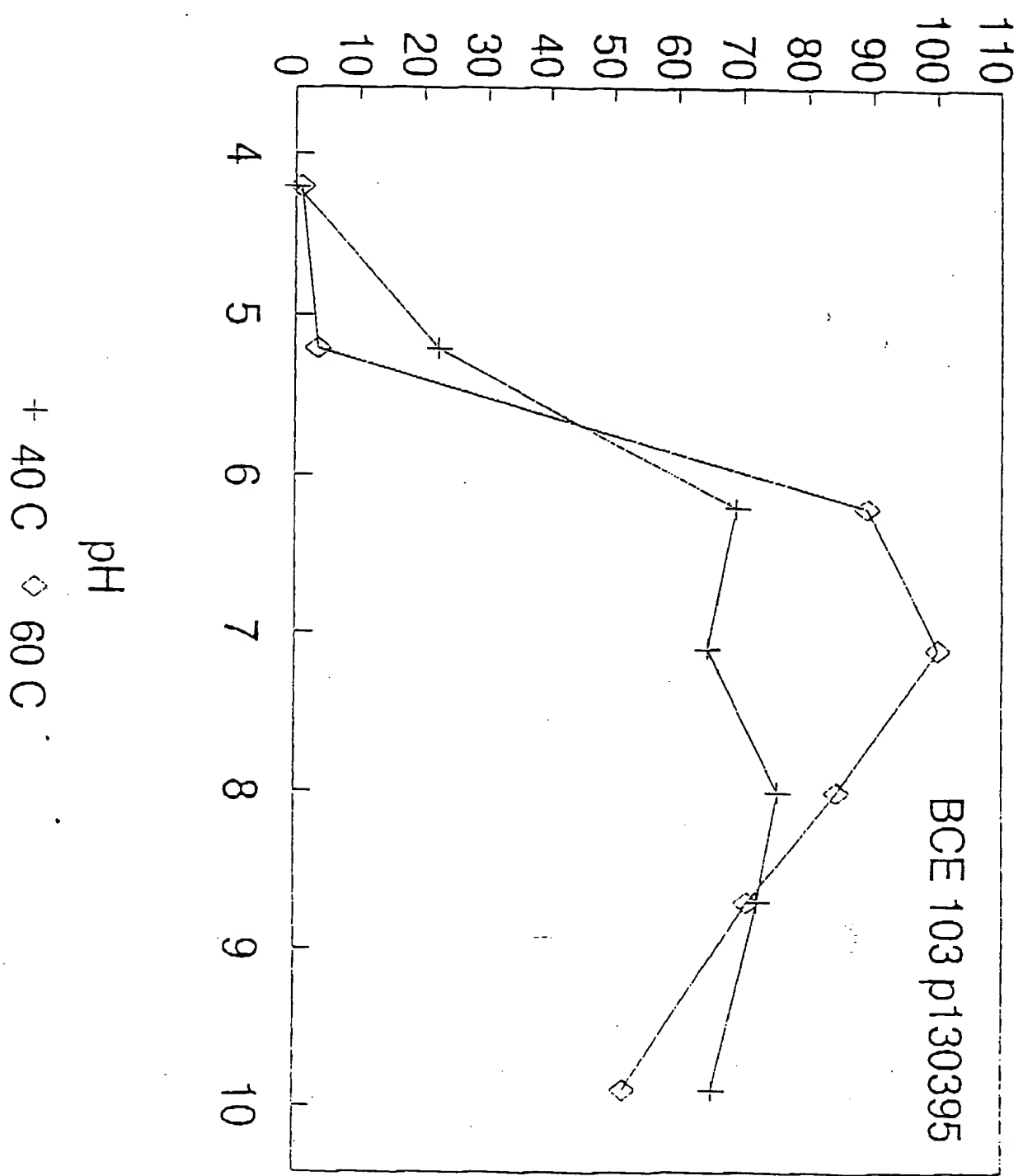


Figure 1